VERIFICATION FOR TRANSLATION

I, Ryoko MIDORIKAWA, a national of Japan, c/o SAEKI & PARTNERS of 4th Floor, Aminosan Kaikan Building, 15-8, Nihonbashi 3-chome, Chuo-ku, Tokyo 103-0027, Japan, do hereby solemnly and sincerely declare:

1) THAT I am well acquainted with the Japanese language, English language, and

2) THAT the attached is a true, accurated and faithful translation into the English language made by me of Japanese Patent Application No. 11-201450/1999 filed to the Japanese Patent Office on July 15, 1999.

Signed this 22 th day of August , 2005.

Ryoko Midorikawa

Ryoko MIDORIKAWA

[Document] Specification

[Titile of the Invention] Novel Nucleic Acid Base Pair Which Improves Enzyme Incorporation Selectivity by Utilizing Steric Hindrance

[Claims]

[Claim 1] A method for constructing selective base pair comprising introducing a group having ability to form steric hindrance in nucleic acid base.

[Claim 2] The method according to claim 1 wherein the group having ability to form steric hindrance is a group to hinder formation of base pair with base part of natural nucleic acid.

[Claim 3] The method according to claim 1 or claim 2 wherein the group having ability to form steric hindrance is dialkylamino group.

[Claim 4] The method according to any of claims 1 - 3 comprising introducing a group to be able to form additional hydrogen bonds.

[Claim 5] The method according to claim 4 wherein group to be able to form additional hydrogen bond is an electron pair of amino group, hydroxyl group, keto group or nitrogen group.

[Claim 6] The method according to any of claims 1 - 5 wherein the base pair is a base pair which can be recognized by polymerase.

[Claim 7] The method according to claim 6 wherein the polymerase is DNA polymerase or RNA polymerase.

[Claim 8] A method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance in the nucleic acid base part.

[Claim 9] A method for designing nucleic acid to construct selective base pair comprising hindering to construct base pair with the natural nucleic acid

base part by utilizing the steric hindrance.

[Claim 10] The method for designing nucleic acid according to claim 8 or claim 9 wherein the nucleic acid base pair is a base pair which can be recognized by polymerase.

[Claim 11] A nucleic acid comprising being designed by the method according to any of claims 8 - 10.

[Claim 12] The nucleic acid according to claim 11 wherein the nucleic acid has base comprising purine derivatives having a group at position-6 which can generate steric hindrance.

[Claim 13] The nucleic acid according to claim 12 wherein the nucleic acid base is 2-amino-6-N,N-dimethylamino-purine.

[Claim 14] The nucleic acid according to claim 11 wherein the nucleic acid has base containing pyridine having hydroxyl group or keto group at position-2.

[Claim 15] The nucleic acid according to claim 14 wherein the nucleic acid base is pyridine-2-one or tautomer thereof.

[Claim 16] The nucleic acid according to any of claims 11 - 15 wherein the nucleic acid is a nucleic acid constructing base pair with its complementary nucleic acid.

[Claim 17] A process for production of the nucleic acid comprising the nucleic acid according to any of claims 11 - 16.

[Claim 18] The process according to claim 17 wherein the nucleic acid is another nucleic acid to construct base pair.

[Claim 19] A codon comprising one or more nucleic acid according to any of claims 11 - 16.

[Claim 20] The codon according to claim 19 wherein the codon encodes amino acids.

[Claim 21] The codon according to claim 20 wherein amino acids are non-natural amino acids.

[Claim 22] A nucleic acid molecule comprising containing the nucleic acid according to any of claims 11 - 16 and natural nucleic acid.

[Claim 23] The nucleic acid molecule according to claim 22 wherein the nucleic acid molecule encodes proteins.

[Claim 24] The nucleic acid molecule according to claim 22 or claim 23 wherein the nucleic acid molecule has whole or part of genetic information of the natural gene.

[Claim 25] A process for production of nucleic acid having complementary strand thereof comprising reacting the polymerase with the nucleic acid according to any of claims 22 - 24.

[Claim 26] The method according to claim 25 wherein the polymerase is DNA polymerase or RNA polymerase.

[Claim 27] A process for production of non-natural nucleic acid comprising inserting or substituting one or more nucleic acid according to any of claims 11 - 16 in the natural nucleic acid.

[Claim 28] The process for production of non-natural nucleic acid according to claim 27 wherein a position, to which the nucleic acid according to any of claims 11 - 16 is inserted or substituted, has a codon unit and the other part has base sequence encoding natural amino acid sequence.

[Claim 29] A process for production of protein having amino acid sequence based on codons of the nucleic acid according to any of claims 22 - 24 or the

non-natural nucleic acid obtained by the method according to claim 27 or claim 28.

[Claim 30] The process for production of protein according to claim 29 comprising being inserted or substituted by the non-natural amino acid in the part or whole of amino acid sequence of natural protein.

[Claim 31] A microorganism which is transformed by non-natural gene which can be produced by the process according to claim 27 or claim 28.

[Claim 32] A method for screening functions of amino acids coded by natural gene comprising using the non-natural gene which can be produced by the method according to claim 27 or claim 28.

[Detailed Description of Invention]

[0001]

[Field of the Invention]

The present invention relates to formation of selective novel artificial nucleic acid base pair by utilizing steric hindrance.

The present invention further relates to replication and transcription of nucleic acid using the novel artificial nucleic acid base pair of the present invention, and a system for protein synthesis or functional nucleic acid. More particularly, the present invention pertains the novel artificial nucleic acid having properties to form selective base pair by applying steric hindrance, a process for production thereof, codon containing the same, nucleic acid molecule containing the same, a process for production of novel protein using the above nucleic acid molecules or non-natural gene.

[0002]

[Related Art]

Genetic information of organisms in the earth are transferred by using nucleic acids comprising of four bases consisting of adenine (A), guanine (G), cytosine (C) and thymine (T) as a gene. Proteins are synthesized according to genetic informations of mRNA which is transcribed from DNA of gene. In that occasion, 64 types of codon consisting of 3 bases ($4^3 = 64$) correspond to 20 types of amino acids.

If novel nucleic acid base (X and Y, in which X and Y form specific base pair) can be created in addition to already known for bases (A, G, C, T), numbers of codon can be increased greatly ($6^3 = 216$). As a result, proteins containing non-natural amino acids can possibly be synthesized by matching the newly created codons with non-natural amino acids [J. D. Bain, et al. Nature, 356, 537-539 (1992)].

[0003]

Heretofore, a pair of isocytosine and isoguanine has been reported as an artificial base pair except for A-T and G-C. Isoguanine tends to form base pair with thymine due to tautomerism of isoguanine [C. Switzer, et al. J. Am. Chem. Soc. 111, 8322-8323 (1989); C. Y. Switzer, et al. Biochemistry 32, 10489-10496 (1993)]. Several novel base pairs have been reported, but there were problems on recognition by polymerase and no practical use has known [J. A. Piccirilli, et al., Nature, 343, 33-37 (1990); J. Horlacher, et al. Proc. Natl. Acad. Sci. USA, 92, 6329-6333 (1995); J. C. Morales, et al., Nature struct. biol., 5, 954-959 (1998)].

[0004]

Nucleic acid molecules having various functions were found by in vitro

selection method [A. D. Ellington, et al. Nature 346, 818-822 (1990); C. Tuerk, et al. Science 249, 505-510 (1990)]. If the novel base pair X-Y hereinabove can be recognized by polymerases such as DNA polymerase, RNA polymerase and reverse transcriptase, the present in vitro selection method using 4 bases can be performed by using 6 bases, then possibility to create nucleic acid molecules having novel function, which could not be practically realized by using 4 bases, can be expected.

Further, creation of novel base pair has expected for treatment of hereditary diseases caused by gene abnormality, in which one or more base in the gene is replaced by different base.

[0005]

[Problems to be Resolved by the Invention]

The present invention provides ideas for selective formation of novel artificial nucleic acid base pair as a result of recognition of base pairing by polymerase such as DNA polymerase by utilizing steric hindrance between base pairs.

An aspect of the present invention is to provide novel artificial nucleic acid base pair which does not form base pair with natural nucleic acid and forms selective base pair in themselves as well as being recognized by various polymerases. Further aspect of the present invention is to provide artificial nucleic acid, codon containing the same, nucleic acid molecule, non-natural gene and application thereof.

[0006]

[Means for Solving the Problems]

The present inventors have studied extensively to create novel

artificial nucleic acid base pairs, which could not form base pair with natural nucleic acid, but could selectively form base pair by themselves and could be recognized by various polymerases, and have found out that formation of nucleic acid base pair with natural nucleic acid could be inhibited by applying steric hindrance of nucleic acid base, and formation of selective base pair between newly designed nucleic acid bases could be made. Further, we have found that such the newly designed nucleic acids could be recognized by various natural polymerases.

For example, in order not to form base pair with thymine but to form steric hindrance with keto group at position-6 of thymine, 2-amino-6-(N,N-dimethylamino) purine (hereinafter designates as X), in which two bulky methyl groups are introduced in amino group at position-6 of 2,6-diaminopurine, is designed. As a result, the X does not form base pair with thymine, but bases such as pyridine-2-one (hereinafter designates as Y), an analog of thymine, in which oxo group at position-6 is replaced by hydrogen atom, can form base pair with X (refer to Fig. 1).

[0007]

Further, we have synthesized DNA oligomer containing 2-amino-6-(N,N-dimethylamino)-9-(2'-deoxy- β -D-ribofuranosyl) purine (hereinafter designates as dX) and 3-(2'-deoxy-5'-triphosphoro- β -D-ribofuranosyl) pyridine-2-one (hereinafter designates as dYTP), and found that dYTP or its ribonucleotide (rYTP) could be incorporated selectively into DNA or RNA as a complementary strand of the above dX.

[0008]

The present invention provides novel artificial nucleic acid base pair

capable of forming selective base pair by utilizing steric hindrance which can be recognized by polymerases such as DNA polymerase, and novel artificial genes.

[0009]

The present invention relates to a method for constructing selective base pair comprising introducing a group having ability to form steric hindrance. More particularly, the present invention relates to a method for constructing selective base pair wherein the said group having ability to form steric hindrance is a group to hinder formation of base pair with base part of natural nucleic acid, and the said base pair can be recognized by polymerase.

Further, the present invention relates to a method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance in the nucleic acid base part. More particularly, the present invention relates to a method for designing nucleic acid to construct selective base pair by utilizing steric hindrance to hinder construction of base pair with natural nucleic acid base part, and the said base pair can be recognized by polymerase.

The present invention relates to a nucleic acid which can construct selective base pair, prepared by introducing a group having ability to form steric hindrance. More particularly, the present invention relates to a nucleic acid and production method thereof, for constructing selective base pair wherein the said group having ability to form steric hindrance to hinder formation of base pair with base part of natural nucleic acid, and the said base pair can be recognized by polymerase.

[0010]

The present invention discloses novel artificial nucleic acid which have similar behavior with nucleic acids containing natural bases and a method for designing such the nucleic acid. The nucleic acid of the present invention can be applied in the similar manner as the natural nucleic acid.

Consequently, the present invention relates to various applications using the nucleic acid of the present invention or nucleic acid designed by the method of the present invention.

More particularly, the present invention relates to a codon comprising one or more nucleic acid designed by the nucleic acid of the present invention or nucleic acid designed by the method of the present invention. The said codon can encode amino acids in the similar manner as the natural nucleic acid. The said amino acids can be non-natural amino acids. Further, the present invention relates to a nucleic acid molecule containing the nucleic acid of the present invention, the nucleic acid designed by the method of the present invention or the nucleic acid of the natural origin. The said nucleic acid molecule can encode proteins in the similar manner as the natural nucleic acid. Further the said nucleic acid molecule can maintain whole or part of genetic informations of the natural gene. Nucleic acid having complementary strand can be prepared by an action of various polymerases on such the nucleic acid molecule. The present invention also relates to such process for production of complementary strands.

[0011]

In addition, the nucleic acid of the present invention or the nucleic acid designed by the method of the present invention can be introduced or substituted to a part of natural gene. Consequently, the present invention

relates to a process for production of non-natural gene comprising introducing or substituting one or more nucleic acid of the present invention or the nucleic acid designed by the method of the present invention into the natural gene. The introduction or substitution can be performed with the codon unit of the present invention as described hereinbefore.

Further, the present invention relates to a process for production of protein having amino acid sequence based on codons of the non-natural gene or the nucleic acid of the present invention. Protein to which non-natural amino acid is introduced or substituted in the part of natural protein can be produced in case that codon containing the nucleic acid of the present invention or the nucleic acid designed by the method of the present invention encodes non-natural amino acid.

[0012]

Consequently, the present invention provides a process for production of novel protein comprising substituting or introducing other natural or non-natural amino acid, preferably non-natural amino acid in a part of natural protein by the method of the present invention. According to this method, functions of amino acids in the protein coded by natural gene can be screened. The present invention also relates to a method for screening function of each amino acid of protein encoded by natural gene.

The present invention also relates to a microorganism transformed by non-natural gene containing the nucleic acid of the present invention or nucleic acid designed by the method of the present invention (hereinafter simply designates as the nucleic acid of the present invention).

Further, since the novel base pair of the present invention does not

constitute base pairing with the natural bases, it is useful for treatment of hereditary diseases caused by gene in which one or more base is replaced by other base. The present invention provides pharmaceutical composition comprising novel base pair or a base in the said base pair.

[0013]

An object of the present invention is to provide artificial nucleic acid which does not form base pair with a base of the natural nucleic acid and can be recognized by polymerase. The conventional artificial nucleic acid has produced by attempting to change at the position of hydrogen bond, as a result, base pairing with a base of the natural nucleic acid could not be hindered substantially as well as showing insufficient base pair selectivity. We have solved such the problem by introducing a group forming the steric hindrance, preferably by introducing a group forming steric hindrance and electrostatic repulsion and having stacking action. The present invention provides novel artificial nucleic acid which can form selective base pairing with artificial nucleic acids themselves.

Consequently, the present invention will be explained more concretely by referring examples hereinbelow, but these examples are illustrated only for the purpose of better understanding of the present invention, and the fact that the present invention is not limited by these examples is obvious according to the technical idea of the present invention explained hereinbefore.

[0014]

A group which forms steric hindrance in the base part of the nucleic acid of the present invention can be a group only hinder hydrogen bonding

with deteriorated base, and is not limited if it does not deteriorate for the properties as a base of nucleic acid. Preferably, the size thereof may be not to hinder formation of base pairs of other bases in the nucleic acid sequence. In addition, it is preferable not to have polar group and activated hydrogen atom, but is not necessary to consider if these polar group and activated hydrogen atom are located in the positions having distance impossible to form hydrogen bonding

Examples of group which forms steric hindrance are, for example, lower alkyl group such as ethyl, isopropyl, isobutyl or t-butyl group, preferably branched lower alkyl group, lower alkoxy group consisting of methyl or these lower alkyl groups, di-lower alkylamino group substituted by methyl or these lower alkyl groups and silyl group substituted by methyl or these lower alkyl groups.

Conventional chemical synthesis can be applied for methods of introducing groups to form steric hindrance in the base.

[0015]

Nucleic acid of the present invention is artificial nucleic acid which can be recognized by polymerase. Examples of polymerase can be any polymerase, preferably DNA polymerase and RNA polymerase. Recent studies on structural analysis of polymerase indicates that interaction of polymerase and nucleic acid is essentially identical with each other. Formation of base pair of the present invention relates to essential nature of polymerase, consequently, the base pair formation of the present invention can be utilized not only for DNA polymerase and RNA polymerase but also for all polymerase including reverse transcriptase.

[0016]

Further, configuration of nucleic acid can be calculated by analysis of molecular configuration or precise determination of distance between atoms. Consequently, by applying these results, chemical structure, which causes steric hindrance in one side and provides one or more hydrogen bonds, preferably two hydrogen bonds in other side, can be designed. Consequently, the present invention includes a method for designing artificial nucleic acid based on steric hindrance of the nucleic acid, preferably steric hindrance in the base part of nucleic acid. In the designing base pair of the present invention, designing based on Watson-Crick type base pair is conventional, but Hoogsteen base pairing may also be applicable.

[0017]

Nucleic acid of the present invention can be a nucleic acid designed by steric hindrance of nucleic acid, preferably designed by steric hindrance in the base part of the nucleic acid, and is preferable to form selective base pairing with each of artificial nucleic acid. Preferably, base pairing of artificial nucleic acid can be recognized by polymerase and more preferably the complementary strand can be constructed similar to the natural nucleic acid by and action of polymerase.

Nucleic acid of the present invention can be synthesized by conventional chemical synthesis but is not limited to that method. Chemical synthesis is exemplified in Fig. 2 and Fig.3.

[0018]

Method for incorporating nucleic acid of the present invention into the nucleic acid sequence can be performed by applying conventional method for

incorporation of natural nucleic acid or by applying similar method thereof. For example, a method using DNA synthesizer, method for using polymerase and point mutation technology can be mentioned. Labeling can also be possible made as same as in the natural nucleic acid.

The present invention also includes nucleic acid which can be used for gene fragment or probe, and include nucleic acid molecule containing the nucleic acid of the present invention. The nucleic acid molecule of the present invention contains one or more nucleic acid of the present invention, and can be a single strand or double strands. Non-natural gene of the present invention includes natural gene in which whole of part of it is replaced by nucleic acid of the present invention, natural gene to which one or more nucleic acid of the present invention is added, or combination thereof. Such the non-natural gene of the present invention can be modified by the same or similar method used for the conventional modification of natural gene.

[0019]

Consequently, nucleic acid molecule or non-natural gene of the present invention can be used for transformation of microorganisms by the same way as in the conventional natural gene by inserting suitable vector or phage and inserted into microorganisms to produce transformant containing the artificial nucleic acid of the present invention.

[0020]

Further, new codon containing nucleic acid of the present invention can be designed. For example, the present novel artificial nucleic acid base is set as X and Y, combination thereof such as XXY, XYX, YXX, a combination by themselves, and AXA, TYT, CGX, ATX, which are combination of base of

natural nucleic acid and artificial base of the present invention. Such codons can be designed. New codons can code natural amino acid, or non-natural amino acid. Further, functions such as transcription, transfer can be coded. Accordingly, the present invention not only provide novel artificial nucleic acid, but also providing possibility of designing completely new genetic code by designing new codon containing nucleic acid of the present invention.

[0021]

As a result of designing t-RNA corresponding to new codon of the present invention, new protein synthesis system can be designed by which large number of amino acid can be utilized. Usable amino acid can be amino acid utilized on protein synthesis on liposome. Consequently, the present invention provides novel protein synthetic system using codon of the present invention.

Heretofore, some amino acids in the natural protein are very difficult to substitute non-natural amino acid, or insertion of non-natural amino acid into the natural protein is also very difficult. According to the protein synthesis system of the present invention, proteins containing desired non-natural amino acid can be produced by substituting or inserting the nucleic acid having codon of desired position into the nucleic acid of the present invention. And such the conversion of amino acid resulted to make screening functions of amino acid in the protein.

[0022]

The present invention will be explained by the following examples in detail.

One of artificial base, 2,6-diaminopurine, could form base pairing with

thymine by hydrogen bonding at position-6 of thymine. In order not to form base pairing 2,6-diaminopurine with thymine, two bulky methyl groups were introduced into the amino group at position-6 of 2,6-diaminopurine for colliding with this group and keto group of thymine at position-6 by steric hindrance, and synthesized to design 2-amino-6-(N,N-dimethylamino) purine (hereinafter a base of which is designated as X) . Accordingly, X could not form base pairing with thymine an analogous base in which oxo group at position-6 of thymine is replaced by hydrogen, pyridine-2-one (hereinafter the base of which is designated as Y) could for base pairing with X (refer to Fig. 1). Lower part of Fig. 1 illustrates binding not to form base pairing these bases X and Y with other bases.

[0023]

In order to examine formation of selective novel nucleic acid base pair by utilizing steric hindrance in the base pair X-Y, primer extension method of DNA and transcriptional reaction synthesizing RNA from DNA were applied. The primer extension method includes annealing template DNA oligomer with a primer oligomer, and adding DNA polymerase and 2'-deoxynucleotide-5'-triphosphate (dNTP) to extend complementary sequence of the template at 3'-terminal of the primer. Klenow fragment, which is deleted 5'-exonuclease from DNA polymerase I, one of DNA polymerase originated from E. coli, and T7 RNA polymerase, RNA polymerase originated from T7 phage, were herein used. Both enzymes are commonly used at present.

[0024]

In order to incorporate X into the template DNA, amidite reagent of

dX was synthesized (refer to Fig. 2). Also template DNA containing dX having base sequences hereinbelow (Template 3, 5, 6, 7, 8 and 9) and template DNA (Template 1, 2 and 4) and their primer (Primer 1, 2 and 3) for use of control experiments were synthesized. Substrates, dYTP and rYTP, were also synthesized (refer to Fig. 3)

5'-terminal of the primer was labeled with ^{32}P using T4 polynucleotide kinase and [α-^{32}P] ATP. Primer labeled with ^{32}P (0.5 μM) and template 1 and 3 (1 μM) and various dNTP (150 μM) were used for primer extension by Klenow fragmentat 17℃ for 30 minutes. Results indicate that a base y was incorporated into complementary strands of A, G and X. To the complementary strand X was incorporated also C and T in addition to Y (refer to Fig. 4).

[0025]

For quantitative analysis of the incorporation, same experiments were conducted by adding only dNTP (150 μM) using primer 2 (1 μM) labeled with ^{32}P at 5'-terminal and template 1, 2 and 3 (2 μM). As a result, Y was incorporated into complementary strands of A, G and X at 78%, 48% and 41%, respectively, and Y, C and T were incorporated into complementary strand of X, at 41%, 9.5% and 13%, respectively. (Refer to Fig. 5)

Since Y was incorporated independently into not only X but also A and G, the following experiments were conducted in order to find out to what strands Y was incorporated when T and C were coexisted.

[0026]

Primer 2 without labeling and template 2 were annealed, and [α-^{32}P] TTP and various amounts of dYTP were added thereto to find out ratio of

inhibition of incorporation of [α-^{32}P] TTP into X by dYTP was investigated. Simultaneously with addition of dATP, effect of the said inhibition on incorporation of A into complementary strand of T next to X was investigated (refer to Fig. 6 A). As a result, when dYTP was added almost equivalent level of [α-^{32}P] TTP, incorporation of [α-^{32}P] TTP into X was inhibited at 50%. Same experiments were conducted by using template 1 and 2 for A and G. dYTP did not inhibit incorporation of [α-^{32}P] TTP into the complementary strand of A and the incorporation of [α-^{32}P] CTP into the complementary strand of G. (Refer to Fig. 6 B and C). Consequently, incorporation of dYTP into A and G was suppressed by coexisting TTP and dCTP.

[0027]

In order to search effect of incorporation of Y, C and T into the complementary strand of X, when two X are presented on the template, ^{32}P labeled primer 3 at 5'-terminal and template 4, 5, 6, 7, 8 and 9 were used for primer extension method. As a result, when two X were continued on the template, polymerase reaction was terminated at the position where two continuous X were existed whenever using any bases. In only the case where another base was incorporated between two X, only Y was incorporated into the complementary strand of the second X in the two X and continued synthesis of complementary strand (refer to Fig. 7).

[0028]

Similarly, a transcription reaction by RNA polymerase using DNA containing X as template was examined. Using template 1-3, promoter region on this strand was duplicated, and the transcription reaction was examined using T7 RNA polymerase by adding [α-^{32}P] ATP (refer to Fig.8, y in the

figure means Y). In case of using template 1 (N = X), a band corresponding to a product as a result of selective incorporation of Y to X is observed. Trace amount of U was incorporated. In the case of template 2 (N = A), not only U but also Y was found to incorporated into the complementary strand of A. In the case of the template 3 (N = G), only C was incorporated, and almost no production as a result of incorporation of Y was observed.

[0029]

Transcription reaction in the presence of rNTP was conducted. Using the forgoing respective template, the transcription reaction was examined using T7 RNA polymerase by adding [α-^{32}P] ATP, then generated full length of RNA was digested completely by RNase T2, nucleotide labeled at 3'-terminal was analyzed by 2-dimention TLC(refer to Fig.9, y in the figure means Y). Result indicates that Y is almost selectively incorporated in case of transcription reaction using the template 1 (N = X) and trace amount of U is detected. In the case of template 2 (N = A), no incorporation of Y is observed.

[0030]

As explained hereinabove, it is revealed that selective formation of base pair which has never achieved in the heretofore reported artificial base pair is feasible by utilizing steric hindrance. Such artificial nucleic acid base pair can be applied to duplication and transcription of nucleic acid and to protein synthesis system or functional nucleic acid.

[0031]

[Examples]

Following examples illustrate the present invention but are not construed as limiting the present invention.

[0032]

Example 1:

Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-[5'-O-dimethoxytrityl-3'-O-[[(diisopropylamino)-2-cyanoethoxy] phosphino]-2'-deoxy-β-D-ribofuranosyl] purine (10)

[0033]

(A) Synthesis of 2-amino-6-(N,N-dimethylamino)-9-(2',3,5'-tri-O-acetyl-β-D-ribofuranosyl] purine (2):

2-amino-6-chloro-9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl) purine (1) [M. J. Robins and B. Uznanski, Can. J. Chem., 59, 2601-2607 (1981)](18.6 mmol, 7.96 g) was dehydrated three times azeotropically with anhydrous pyridine, and dissolved in anhydrous pyridine (180 ml), then dimethylamine hydrochloride (55.8 mmol, 4.55 g) and diisopropylethylamine (74.4 mmol, 7.96 g) were added thereto with stirring at room temperature. The mixture was stirred at room temperature for 15 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue, and the organic layer was washed 3 times with water, 2 times with 5% aqueous sodium hydrogen carbonate, once with water and 2 times with 10% aqueous citrate solution, then the organic layer was dried with magnesium sulfate, and dried in vacuo after filtration. The residue was treated with azeotropic distillation with toluene until no odor of pyridine was noted, the product was purified by silica-gel chromatography (dichloromethane-ethanol) to obtain the product (2) 5.42 g (12.4 mmol)(67%).

^{1}H-NMR (500.13 MHz, $CDCl_3$) δ : 7.56 (s, 1H, H8), 6.02 (d, 1H, H1', J = 5.0

Hz), 5.95 (dd, 1H, H2', J = 5.0 Hz), 5.79 (t, 1H, H3', J = 5.0 Hz), 4.69 (s, 2H, 2-NH_2), 4.42-4.45 (m, 1H, H4'), 4.34-4.40 (m, 2H, H5', H5"), 3.43 (br, 6H, N-CH_3), 2.13, 2.10, 2.08 (s, 3H, Ac).

[0034]

(B) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-[2',3',5'-tri-O-acetyl-β-D-ribofuranosyl] purine (3)

A compound (2) obtained in the above (A) (10 mmol, 4.36 g) was azeotropically dehydrated three times and dissolved in anhydrous pyridine (180 ml). Under stirring at room temperature, benzoyl chloride (15 mmol, 1.74 ml) was added and the mixture was stirred at room temperature for 14 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue, and the organic layer was washed 2 times with 5% aqueous sodium hydrogen carbonate and once with water. The organic layer was dried with magnesium sulfate and filtered, then concentrated in vacuo. The residue was treated by azeotropic distillation until the residue showed no odor of pyridine. The residue was purified by silica-gel column chromatography (hexane-dichloroethane) to obtain the product (3) 3.53 g (6.53 mmol) (65%).

^{1}H-NMR (500.13 MHz, $CDCl_3$) δ : 8.46 (s, 1H, H8), 7.96 (d, 2H, Bz-m, J = 10.0 Hz), 7.75 (s, 1H, NHBz), 7.55 (dd, 1H, Bz-p, J = 7.5 Hz), 7.48 (t, 2H, H Bz-o, J = 7.5), 6.08 (d, 1H, H1', J = 3.0 Hz), 5.96-6.01 (m, 2H, H2', H3'), 4.39-4.50 (m, 3H, H4', H5', H5"), 3.48 (br, 6H, N-CH_3), 2.15 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 3H, Ac).

[0035]

(C) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(β -D-ribofuranosyl) purine (4)

To a compound (3) obtained hereinabove (B) (6.53 mmol, 3.53 g) , pyridine-methanol-water (65 : 30 : 5) 50 ml was added and stirred in the ice-water bath. 2M sodium hydroxide-pyridine-methanol-water (65 : 30 : 5) 50 ml was added and stirred for 15 minutes in the ice-water bath. After confirming completion of the reaction, ammonium chloride (5.21 g) was added to the reaction mixture and concentrated in vacuo until volume reached up to 40 ml. Chloroform was added to the solution, and the organic layer was extracted. Then the aqueous layer was extracted twice with chloroform-pyridine. The organic layer was collected and dried with magnesium sulfate. The filtrate was concentrated up to volume of 10 ml in vacuo. Toluene was added thereto and concentrated in vacuo to precipitate crystals. Crystals were collected by filtration and dried in vacuo at 90℃ to obtain the product (4) 2.87 g.

^{1}H-NMR (500.13 MHz, DMSO-d_6) δ : 8.28 (s, 1H, H8), 7.92 (dd, 2H, Bz-m, J = 7.0 Hz), 7.57 (dd, 1H, Bz-p, J = 7.3 Hz), 7.49 (t, 2H, H Bz-o, J = 7.5), 5.91 (d, 1H, H1', J = 4.0 Hz), 5.48 (d, 1H, OH, J = 5.5 Hz), 5.15 (d,1H, OH, J = 4.0 Hz), 5.04 (t, 1H, OH, J = 1.0 Hz), 4.56 (t, 1H, H2', J = 10.0 Hz), 4.17 (d, 1H, H3', J = 3.0 Hz), 3.93 (d, 3H, H4', J = 3.5 Hz), 3.63-3.65 (m, 1H, H5'), 3.52-3.56 (m, 1H, H5"), 3.48 (br, 6H, N-CH_3).

[0036]

(D) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(3',5'-O-tetraisopropyldisiloxanyl- β -D-ribofuranosyl) purine (5)

A compound (4) obtained in the above (C) (5.0 mmol, 2.07 g) was dehydrated three times azeotropically with anhydrous pyridine and dissolved in anhydrous pyridine (50 ml), then 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (5.5 mmol, 1.76 ml) was added and stirred at room temperature for 14 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue. The organic layer was washed twice with 5% aqueous sodium hydrogen carbonate and once with saturated sodium chloride solution. The organic layer was dried with magnesium sulfate, filtered and dried in vacuo. Azeotropic treatment was repeated until no odor of pyridine in the residue was noted. Then the residue was purified by silica-gel chromatography (dichloromethane-methanol) to obtain the product (5) 2.63 g (4.0 mmol)(80%). ^{1}H-NMR (500.13 MHz, $CDCl_3$) δ : 8.22 (s, 1H, H8), 7.89 (d, 2H, Bz-m, J = 5.0 Hz), 7.80 (s,1H, NHBz), 7.55 (dd, 1H, Bz-p, J = 7.5 Hz), 7.48 (t, 2H, Bz-o, J = 7.5 Hz), 5.91 (s, 1H, H1'), 4.84 (dd, 1H, H3', J = 5.5 Hz), 4.50 (d, 1H, H2', J = 5.5 Hz), 4.07-4.20 (m, 2H, H4', H5'), 4.06 (d, 1H, H5", J = 13.0 Hz), 3.48 (br, 6H, N-CH_3), 0.95-1.08 (m, 28H, iPr).

[0037]

(E) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(2'-O-phenoxythiocarbonyl-3',5'-O-tetraisopropyldisiloxanyl-β-D-ribofuranosyl) purine (6)

The compound (5) obtained in the above (D) (3.98 mmol, 2.61 g) was dehydrated azeotropically three times with anhydrous toluene and dissolved in anhydrous dichloromethane (40 ml). 1-methyimidazole (7.96 mmol, 0.64 ml)

and chlorothio carbonate phenyl (5.57 mmol, 0.77 ml) were added with stirring at room temperature, then stirred at room temperature for 16 hours. After confirming completion of the reaction by TLC, 5% aqueous sodium hydrogen carbonate was added to the reaction mixture. After extracted the organic layer, the organic layer was washed once with aqueous 5% sodium hydrogen carbonate, once with water, twice with aqueous 10% citrate solution and once with water, in this order, the organic layer was dried with magnesium sulfate, filtered and dried in vacuo. The residue was purified by silica-gel column chromatography (dichloromethane-methanol) to obtain the product (6) 2.96 g (3.73 mmol) (94%).

^{1}H-NMR (500.13 MHz, DMSO-d_6) δ : 8.17 (s,1H, H8), 7.87 (d, 2H, Bz-m, J = 3.0 Hz), 7.79 (s, 1H, NHBz), 7.55 (t, 1H, Bz-p, J = 7.5 Hz), 7.47 (t, 2H, H Bz-o, J = 7.5 Hz), 7.41 (d, 2H, PhO-o, J = 7.5 Hz), 7.29 (t, 2H, PhO-m, J = 7.5 Hz), 7.13 (d, 1H, PhO-p, J = 10.0 Hz), 6.39 (d, 1H, H2', J = 5.0 Hz), 6.11 (s, 1H, H1'), 5.14-5.17 (m, 1H, H3'), 4.23-4.26 (m, 1H, H5'), 4.07-4.12 (m, 1H, H4', H5"), 3.48 (br, 6H, N-CH_3), 0.99-1.15 (m, 28H, iPr).

[0038]

(F) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(2'-deoxy-3',5'-O-tetraisopropyldisiloxanyl-β-D-ribofuranosyl) purine (7)

The compound (6) obtained in the above (E) (3.73 mmol, 2.96 g) was dehydrated azeotropically three times with anhydrous toluene, and dissolved in anhydrous toluene (88 ml). 2,2'-azo-bis-isobutyronitrile (0.746 mmol, 122 mg) was added thereto with stirring at room temperature and added argon gas with bubbling for 1 hours at room temperature. Thereto was added

tributyltin hydride (5.60 mmol, 1.51 ml) and stirred at 75℃ for 3.5 hours. After confirming completion of the reaction by TLC, the reaction mixture was concentrated in vacuo. The residue was purified by silica-gel column chromatography (dichloromethane-methanol) to obtain the product (7) 2.27 g (3.55 mmol) (95%).

^{1}H-NMR (500.13 MHz, $CDCl_3$) δ : 8.24 (s, 1H, H8), 7.90 (d, 2H, Bz-m, J = 5.0 Hz), 7.83 (s, 1H, NHBz), 7.54 (t, 1H, Bz-p, J = 7.5 Hz), 7.48 (t, 2H, H Bz-o, J = 7.5 Hz), 6.29 (dd, 1H, H1', J = 7.5 Hz), 4.80-4.83 (m, 1H, H3'), 3.97-4.07 (m, 2H, H5', H5"), 3.86-3.88 (m, 1H, H4'), 3.50 (br, 6H, N-CH_3), 2.68-2.71 (m, 1H, H2'), 2.59-2.63 (m, 1H, H2"), 1.03-1.09 (m, 28H, iPr).

[0039]

(G) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(2'-deoxy- β -D-ribofuranosyl) purine (8)

The compound (7) obtained in the above (F) (3.55 mmol, 2.27 g) was added to 1M solution of tetrabutylammonium fluoride - tetrahydrofuran (14 ml) and stirred at room temperature for 15 minutes. After confirming completion of the reaction by TLC, the reaction mixture was concentrated in vacuo. The residue was dissolved in chloroform, washed with small amount of water, and the aqueous layer was extracted 4 times with chloroform. The organic layer was dried with magnesium sulfate. The filtrate was concentrated in vacuo and the residue was treated azeotropically with toluene until no odor of pyridine was noted. The residue was dissolved in methanol. Dichloromethane was added dropwise little at a time to crystallize the product. The crystals were collected by filtration and dried in vacuo to obtain the product (8) 0.964 g (2.42 mmol) (68%).

^{1}H-NMR (500.13 MHz, DMSO-d_6) δ : 8.23 (s,1H, H8), 7.84 (d, 2H, Bz-m, J = 7.5 Hz), 7.50 (t, 1H, Bz-p, J = 7.3 Hz), 7.42 (t, 2H, H Bz-o, J = 7.5 Hz), 6.25 (t, 1H, H1', J = 7.0 Hz), 5.21 (s,1H, OH), 4.89 (s, 1H, OH), 4.33 (s, 1H, H3'), 3.77 (s, 1H, H4'), 3.50-3.53 (m, 1H, H5'), 3.43-3.46 (m, 1H, H5"), 3.48 (br, 6H, N-CH_3), 2.56-2.61 (m, 1H, H2'), 2.16-2.18 (m, 1H, H2").

[0040]

(H) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(5'-O-dimethoxytrityl-2'-deoxy-β-D-ribofuranosyl) purine (9)

The compound (8) obtained in the above (G) (1.47 mmol, 0.585 g) was azeotropically dehydrated three times with anhydrous pyridine and dissolved in anhyride pyridine (10 ml). 4,4'-dimethoxy tritylchloride (1.61 mmol, 547 mg) was added with stirring at room temperature, then stirred at room temperature for 1.5 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue and the organic layer was washed twice with aqueous 5% sodium hydrogen carbonate and once with water, then the organic layer was dried with magnesium sulfate, filtered and concentrated in vacuo. The residue was azeotropically distilled with toluene until no odor of pyridine was noted, and was purified by silica-gel column chromatography (dichloromethane - methanol - 0.5% triethylamine) to obtain the product (9) 0.99 g (1.41 mmol) (96%).

^{1}H-NMR (270.16 MHz, $CDCl_3$) δ : 8.20 (s,1H, H8), 7.79 (s, 1H, NHBz), 7.77 (d, 2H, Bz-m, J = 1.4 Hz), 7.76 (d, 1H, Bz-p, J = 3.5 Hz), 7.14-7.51 (m, 11H, H Bz-o, DMTrI, 6.72 (dd, 4H, DMTr), 6.45 (t, 1H, H1', J = 6.5 Hz), 4.78 (m, 1H,

H3'), 4.14 (m, 1H, H4'), 3.74 (s, 6H, OCH_3), 3.50 (br, 6H, N-CH_3), 3.39-3.47 (m, 1H, -H5'), 3.30-3.33 (m, 1H, H5"), 2.80-2.85 (m, 2H, H2', H2").

[0041]

(I) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-[5'-O-dimethoxytrityl-3'-O-[[(diisopropylamino)-2-cyanoethoxy] phosphino]-2'-deoxy-β-D-ribofuranosyl] purine (10)

The compound (9) (0.864 mmol, 0.605 g) was azeotropically distilled three times with anhydrous pyridine and twice with anhydrous tetrahydrofuran and dissolved in anhydrous tetrahydrofuran (6 ml). N,N-diisopropylethylamine (2.59 mmol, 0.452 ml) and chloro-2-cyanoethoxy-N,N-diisopropyl-aminophosphine (1.73 mmol, 0.385 ml) were added with stirring at room temperature and further stirred at room temperature for 2 hours. After confirming completion of the reaction by TLC, anhydrous methanol was added to the reaction mixture to terminate the reaction. Ethyl acetate was added to the reaction mixture, and the organic layer was washed once with 5% aqueous sodium hydrogen carbonate and three times with saturated aqueous sodium chloride solution. The organic layer was dried with anhydrous sodium sulfate and concentrated in vacuo after filtration. The residue was purified by silica-gel column chromatography (dichloromethane -methanol - 2% triethylamine), and dissolved in small volume of chloroform, and reprecipitated by adding hexane to obtain the product (10) 0.574 g (0.638 mmol) (74%).

^{1}H-NMR (270.16 MHz, $CDCl_3$) δ : 8.14 (s, 1H, H8), 8.13 (s, 1H, H8), 7.72 (s, 1H, NHBz), 7.65-7.70 (m, 2H, Bz-m), 7.16-7.47 (m, 12H, H Bz-p,o, DMTr), 6.70-6.75 (m, 4H, DMTr), 6.27-6.41 (m, 1H, H1'), 4.63-4.80 (m, 1H, H3'),

4.20-4.27 (m, 1H, H4'), 3.74 (s, 6H, OCH_3), 3.24-3.72 (m, 10H, H5', H5", $NCH(CH_3)_2$, N-CH_3), 2.83-3.00 (m, 1H, H2'), 2.40-2.64 (m, 5H, H2", OCH_2CH_2CN), 1.06-1.19 (m, 12H, $NCH(CH_3)_2$).

31P-NMR (109.36 MHz, $CDCl_3$) δ : 149.25.

[0042]

Example 2:

Synthesis of primer and template

Following primer and template were synthesized conventionally by using DNA/RNA synthesizer Type 392, The Perkin-Elmer, Applied Biosystems Div., and cyanoethylamidide reagents of dA, dC, dG and dT, which were available from The Perkin-Elmer, and dX of cyanoethylamidite reagent hereinbefore.

Proviso that in a synthesis of oligomer containing dX, removal of protective group for amino group of dX, i.e. benzoyl group, could not completely be performed by conventional condition using conc. ammonia at 55℃ for overnight, consequently, treatment for removal of the protective group was performed under the condition at 80℃ with conc. ammonia for 10 hours.

[0043]

Primer 1: dcgactcactataggg

Primer 2: dctatagggaggaga

Primer 3: dgcctagttgtaccg

Template 1: dtgctctatcttcctccctatagtgagtcgtattat

Template 2: dtgctctgtcttcctccctatagtgagtcgtattat

Template 3: dtgctctxtcttcctccctatagtgagtcgtattat

Template 4: dagctgtgtgtgtctccggtacaactaggc

Template 5: dagctxtgtgtgtctccggtacaactaggc

Template 6: dagctxxgtgtgtctccggtacaactaggc

Template 7: dagctxtxtgtgtctccggtacaactaggc

Template 8: dagctxtgxgtgtctccggtacaactaggc

Template 9: dagctxtgtxtgtctccggtacaactaggc

[0044]

Example 3:

Synthesis of 3-(2'-deoxy-5'-O-triphosphoryl- β -D- ribofuranosyl) pyridine-2-one (dYTP) (16)

(A) Synthesis of 3-(3',5'-O-tetraisopropyldisiloxanyl- β -D- ribofuranosyl) pyridine-2-one (12)

3-(β -D- ribofuranosyl) pyridine-2-one (11) [J. Matulic-Adamic and L. Beigelman, Tetrahedron Lett., 38, 203-206 (1997)] (2.29 mmol, 520 mg) was azeotropically dehydrated three times with anhydrous pyridine and was dissolved in anhydrous pyridine (23 ml). 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.52 mmol, 0.81 ml) was added with stirring at room temperature and further stirred at room temperature for overnight. After confirming completion of the reaction by TLC, water was added to the reaction mixture to terminate the reaction and concentrated in vacuo. The residue was dissolved in chloroform. The organic layer was washed twice with aqueous 5% sodium hydrogen carbonate and once with aqueous saturated sodium chloride solution. The organic layer was dried with magnesium sulfate, and filtered. The filtrate was concentrated in vacuo. The

residue was purified by using silica-gel chromatography (dichloromethane - methanol) to obtain the product (12) 442 mg (0.94 mmol) (41%).

^{1}H-NMR (270.06 MHz, $CDCl_3$) δ : 13.07 (br, 1H, NH), 7.78 (d, 1H, H4, J = 6.8 Hz), 7.37 (d, 1H, H6, J = 4.6 Hz), 6.29 (t, 1H, H5, J = 6.6 Hz), 5.07 (s, 1H, H1'), 4.01-4.30 (m, 5H, H2', H3', H4', H5', H5"), 0.83-1.10 (m, 28H, iPr).

[0045]

(B) Synthesis of 3-(2'-O-imidazothiocarbonyl-3',5'-O-tetraisopropyldisiloxanyl-β-ribofuranosyl) pyridine-2-one (13)

The compound (12) (0.94 mmol, 442 mg) obtained in the above (A) was dehydrated azeotropically three times with anhydrous toluene, dissolved in anhydrous DMF (9 ml). Thiocarbonylimidazolide (2.24 mmol, 401 mg) was added under stirring at room temperature, then the reaction mixture was stirred at room temperature for 7 hours. After confirming completion of the reaction by TLC, ethyl acetate was added to the reaction mixture. The organic layer was washed twice with water, dried with magnesium sulfate, and the filtrate was concentrated in vacuo. The residue was purified by using silica-gel column chromatography (dichloromethane - methanol) to obtain the product (13) 434 mg (0.749 mmol) (80%).

^{1}H-NMR (270.06 MHz, $CDCl_3$) δ : 13.40 (br, 1H, NH), 8.44 (s, 1H, imidazolide), 7.84 (d, 1H, H4, J = 6.8 Hz), 7.73 (s, 1H, imidazolide), 7.33 (d,1H, H6, J = 6.5 Hz), 7.07 (s, 1H, imidazolide), 6.34 (t, 1H, H5, J = 6.8 Hz), 6.23 (d, 1H, H2', J = 5.1 Hz), 5.25 (s, 1H, H1'), 4.46-4.52 (m, 1H, H3'), 4.25-4.29 (m, 1H, H5'), 4.03-4.09 (m, 2H, H4', H5"), 0.87-1.09 (m, 28H, iPr).

[0046]

(C) Synthesis of 3-(2'-deoxy-3',5'-O-tetraisopropyldisiloxanyl-β-ribofuranosyl) pyridine-2-one (14)

The compound (13) (0.749 mmol, 434 mg) obtained in the above (B) was dehydrated azeotropically three times with anhydrous toluene, added ammonium sulfate (8.4 mg), dissolved in hexamethyldisilazane (12.6 ml) and refluxed for 1 hour. The reaction mixture was concentrated in vacuo, dehydrated azeotropically three times with anhydrous toluene, added azobisisobutyronitrile (83.5 mg) and dissolved in anhydrous toluene (16.8 ml). Tributyltin hydride (0.821 ml) was added to the reaction mixture and refluxed for 1 hour. After confirming completion of the reaction by TLC, the reaction mixture was concentrated in vacuo. The residue was purified by using silica-gel column chromatography (dichloromethane - methanol) to obtain the product (14) 0.268 g (0.591 mmol) (79%).

^{1}H-NMR (270.06 MHz, $CDCl_3$) δ : 13.07 (br, 1H, NH), 7.72 (d, 1H, H4, J = 7.0 Hz), 7.31 (d, 1H, H6, J = 6.5 Hz), 6.29 (t, 1H, H5, J = 6.6 Hz), 5.20-5.25 (m, 1H, H1'), 4.37-4.40 (m, 1H, H3'), 3.97-4.12 (m, 2H, H5', H5"), 3.80-3.84 (m, 1H, H4'), 2.26-2.36 (m, 1H, H2'), 1.77-1.86 (m, 1H, H2"), 0.90-1.09 (m, 28H, iPr).

[0047]

(D) Synthesis of 3-(2'-deoxy-β-D-ribofuranosyl) pyridine-2-one (15)

The compound (14) (0.089 mmol, 42 mg) obtained in the above (C) was dehydrated azeotropically three times with anhydrous toluene, added 1 M tetramethyl ammoniumfluoride/THF solution (0.5 ml) and stirred at room temperature for 2 hours. After confirming completion of the reaction by TLC, acetic acid (0.08 ml) was added thereto and concentrated in vacuo. The residue was dissolved in water, washed three times with ethyl acetate, and

the aqueous layer was concentrated in vacuo. The residue was purified by using reverse phase silica-gel chromatography to obtain the product (15) 10.4 mg (0.047 mmol) (52%).

^{1}H-NMR (270.06 MHz, $CDCl_3$) δ : 7.77 (d, 1H, H4, J = 3.8 Hz), 7.36 (d, 1H, H6, J = 3.5 Hz), 6.41 (t, 1H, H5, J = 3.6 Hz), 5.01-5.17 (m, 1H, H1'), 4.29-4.31 (m, 1H, H3'), 3.93-3.95 (m, 1H, H4'), 3.62-3.70 (m, 2H, H5', H5"), 2.31-2.35 (m, 1H, H2'), 1.89-1.95 (m, 1H, H2").

[0048]

(E) Synthesis of 3-(2'-deoxy-5'-O-triphosphoryl- β -D-ribofuranosyl) pyridine-2-one (16)

The compound (15) (0.059 mmol, 13.4 mg) obtained in the above (D) was dehydrated azeotropically three times with anhydrous toluene, dissolved in trimethyl phosphate (0.2 ml), added phosphorus oxychloride (0.065 mmol, 7.1 μ l) under ice-cooling and stirred for 7 hours under ice-cooling. After confirming completion of the reaction by TLC, well mixed solution of 0.5 M bistributylammonium pyrophosphate-DMF solution and tributylamine (70.2 μ l) was immediately added and stirred well under ice-cooling for 30 minutes. 1 M triethylammonium bicarbonate (0.35 ml) was added to the reaction mixture to terminate the reaction and concentrated in vacuo. The residue was dissolved in water and charged on a column of DEAE-Sephadex chromatography (15 $\times$300 mm) and eluted by gradient elution with 50 mM - 1 M triethylammonium bicarbonate. A fraction eluted at 0.53 - 0.59 M was collected and lyophilized.

Structure was confirmed by MS(ESI-), ^{1}H-NMR and ^{31}P-NMR. Sodium salt was prepared by treating with Dowex 50Wx8 column chromatography.

[0049]

MS(ESI-): ($M-H^-$) 449.9.

^{1}H-NMR (270.06 MHz, $CDCl_3$) δ : 7.83 (d, 1H, H4, J = 4.9 Hz), 7.35 (d, 1H, H6, J = 4.9 Hz), 6.51 (t, 1H, H5, J = 4.9 Hz), 5.17 (t, 1H, H1', J = 5.0 Hz), 4.56 (br, 1H, H3'), 4.06 (br, 1H, H4'), 3.99 (br, 2H, H5', H5"), 2.19-2.33 (m, 1H, H2'), 1.81-1.98 (m, 1H, H2").

^{31}P-NMR (109.36 MHz, D_2O) δ : -10.3 (m, 2P, P^1, P^3), -22.7 (m, 1P, P^2).

UV (10 mM phosphate buffer pH 7.0): λ max = 298 nm ($\varepsilon = 7.6 \times 10^3$), 226 nm ($\varepsilon = 7.0 \times 10^3$), λ min = 247 nm, 211 nm.

[0050]

Example 4 :

5'-^{32}P labeling of primer

Primer 1-4 (ca. 1 nmol), 10x polynucleotide kinase buffer (TAKARA) 2 μl, [$\gamma - ^{32}$P]−dATP (ca. 1.1 TBq/mmol) 2 μl, and polynucleotide kinase (10 unit/μl, TAKARA) 2 μl were added into a tube 0.5 ml. The mixture, total 20 μl, was incubated at 37℃ for 40 minutes. The reaction was terminated by adding 10 M urea BPB dye 10 μl, treated at 75℃ for 5 minutes, then electrophoresed using 20% polyacrylamide 7M urea gel electrophoresis (10 cm × 10cm). Main band detected by UV (254 nm) was cut out, transferred to 1.5 ml tube, adding 450 μl of sterilized water and stirred at 37℃ for 12 hours. Supernatant obtained by light centrifugation was transferred to the different tube, added glycogen 1 μl, 3M sodium acetate 40 μl and ethanol 1 ml were added. The mixture was shaken well, thereafter allowed to stand at -30℃ for 1 hour. Then it was centrifuged at -5℃, under 13,000 rpm for 1 hours. The thus obtained precipitate was rinsed with 70% ethanol and dried by using

centrifugal evaporator for 30 minutes. Sterilized water 40 μl was added and kept at 75℃ for 5 minutes, thereafter quantitated at UV (260 nm).

[0051]

Example 5:

Single nucleotide insertion reaction and primer extension reaction using Klenow fragment

5'-^{32}P labeled primer, template and 10 x Klenow fragment buffer (TAKARA) 1 μl were added to the 0.5 ml tube, adjusted total volume to 7 μl, and annealed at 95℃ for 3 minutes, at 40℃ for 3 minutes and at 4℃ for 7 minutes. dNTP 1 μl, Klenow fragment (1 unit/ml, For Sequencing, TAKARA) 2 μl were added, adjusted to total volume to 10 μl and incubated for the fixed time at 17℃. The reaction was terminated by adding 10 M urea BPB dye 5 μl, heated at 75℃ for 5 minutes, and electrophoresed with 20% polyacrylamide gel with 7M urea gel electrophoresis. The result was analyzed using imaging plates (Phosphoroimager analysis).

Results are shown in Fig. 4, Fig. 5 and Fig. 7. Single nucleotide insertion reaction is shown in Fig. 5 and primer extension reaction is shown in Fig. 4 and Fig. 7, respectively.

[0052]

Example 6:

Inhibition experiment for primer extension reaction using Klenow fragment

Primer, template and 10x Klenow fragment buffer (TAKARA) 1 μl were added to 0.5 ml tube, and total volume was adjusted to 7 μl, and

annealed at 95℃ for 3 minutes, at 40℃ for 3 minutes and at 4℃ for 7 minutes. [α -^{32}P] TTP or [α -^{32}P] dCTP and dYTP were added to final concentration for each level, and Klenow fragment (1 unit/ml, For Sequencing, TAKARA) 2 μ l was added, adjusted to total volume to 10 μ l and incubated for the fixed time at 17℃. The reaction was terminated by adding 10 M urea BPB dye 5 μ l, kept at 75 ℃ for 5 minutes, and electrophoresed with 20% polyacrylamide gel with 7M urea gel electrophoresis. The result was analyzed using imaging plates (Phosphoroimager analysis). Result is shown in Fig. 6.

[0053]

Example 7:

Transcription by T7 RNA polymerase

Template DNA 1 μ M, in which promoter region has duplicated strands, and T7 RNA polymerase 2.5 units were added to a solution containing 2mM rNTP, [α -^{32}P] ATP 0.1 μ Ci/ μ l [40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 10 mM rGMP], and incubated for 3 hours. After the reaction, 10 M urea dye was added and kept at 75℃ for 3 minutes, then electrophoresed with 20% polyacrylamide gel. The product was analyzed. Result is shown in Fig. 8.

[0054]

Example 8:

Transcription using T7 RNA polymerase

Reaction was performed as same as in example 7. The generated RNA was isolated by gel electrophoresis. RNA was digested by 0.75 units RNase T2. Each nucleotide was separated using 2-dimension TLC and each ratio was calculated. Result is shown in Fig. 9.

[0055]

[Effect of the Invention]

The present invention indicates that selective base pair formation which could never be achieved by the heretofore reported artificial base pair, can be realized by utilizing steric hindrance. By utilizing the method of the present invention, artificial nucleic acid base pair of the present invention can be applied on replication and transcription of nucleic acid, and protein synthetic system or functional nucleic acid.

[Brief Description of Drawings]

[Fig. 1] Fig. 1 shows novel artificial nucleic acid base pair (X-Y) by utilizing steric hindrance of the present invention.

[Fig. 2] Fig. 2 shows synthetic scheme for amidite reagent for dX of nucleic acid having base X of the present invention.

[Fig. 3] Fig. 3 shows synthetic scheme of dYTP of nucleic acid having base Y of the present invention.

[Fig. 4] Fig. 4 shows 20% polyacrylamide 7M urea gel electrophoresis of primer extension reaction by Klenow fragment using 5'-terminal ^{32}P labeled primer 1 (0.5 μM) and template 1, 3 (1 μM) and various dNTP (150 μM). Reaction was performed at 17℃ for 30 minutes.

[Fig. 5] Fig. 5 shows 20% polyacrylamide 7M urea gel electrophoresis of single nucleotide insertion reaction by Klenow fragment using 5'-terminal ^{32}P labeled primer 2 (1 μM) and template 1, 2, 3 (2 μM) and various dNTP (150 μM). Reaction was performed at 17 ℃ for 30 minutes. A is electrophoretic pattern with 29% polyacrylamide 7M urea electrophoresis and B is a graph of the result.

[Fig. 6] Fig. 6 shows inhibitory experiment by dYTP on primer extension reaction by Klenow fragment using primer 2, template 1, 2, 3 and [α-^{32}P]TTP or [α-^{32}P]dCTP.

A: Primer extension reaction by Klenow fragment was performed at 17℃ for 30 minutes. Primer 2 (1 μ M), template 3 (2 μ M) and [α-^{32}P]TTP (150 μ M) are used.

dYTP, 0, 50, 150, 300 and 500 μ M are added.

Right lane 5: same experiment was performed by adding dATP (300 μ M)

B: Primer extension reaction by Klenow fragment was performed at 17℃ for 10 minutes. Primer 2 (1 μ M), template 1 (2 μ M) and [α-^{32}P]TTP (50 μ M) are used.

dYTP, 0, 20, 100, 500 and 1000 μ M are added.

Right lane 5: same experiment was performed by adding dATP (300 μ M)

C: Primer extension reaction by Klenow fragment was performed at 17℃ for 30 minutes. Primer 2 (1 μ M), template 2 (2 μ M) and [α-^{32}P]TTP (50 μ M) are used.

dYTP, 0, 20, 100, 500 and 1000 μ M are added.

Right lane 5: same experiment was performed by adding dATP (300 μ M)

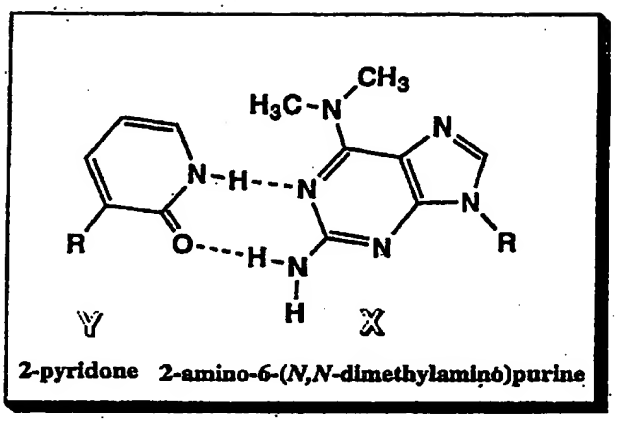
[Fig. 7] Fig. 7 shows primer extension reaction by Klenow fragment using 5'-terminal ^{32}P labeled primer 3(0.33 μ M) and template 4, 5, 6, 7, 8, and 9 (1 μ M) and various dNTP (150 μ M). Reaction was performed at 17℃ for 60 minutes.

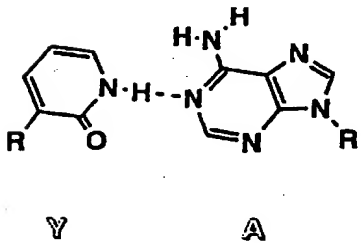
[Fig. 8] Fig. 8: Electrophoresis of RNA generated by transcription of T7 RNA polymerase using various rNTPs and template 1-3 and [α-^{32}P]ATP. y in Fig. 8 is identical to Y in the specification and other figures.

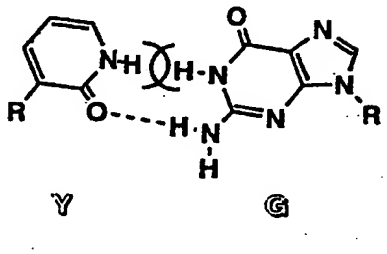
[Fig. 9] Fig. 9: Transcription was performed similar to the case in Fig. 8 with rNTP and generated RNA was purified by electrophoresis and digested by RNase T2. Resulted product was analyzed by 2-dimension TLC. y in Fig. 9 is identical to Y in the specification and other figures.

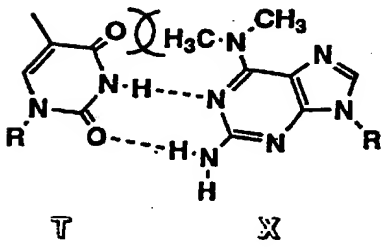
[Document] Drawings

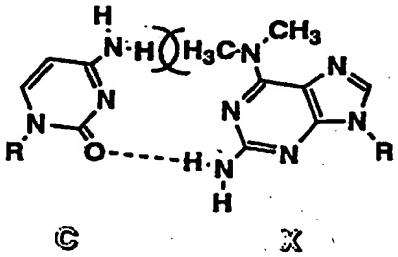
[Fig. 1]





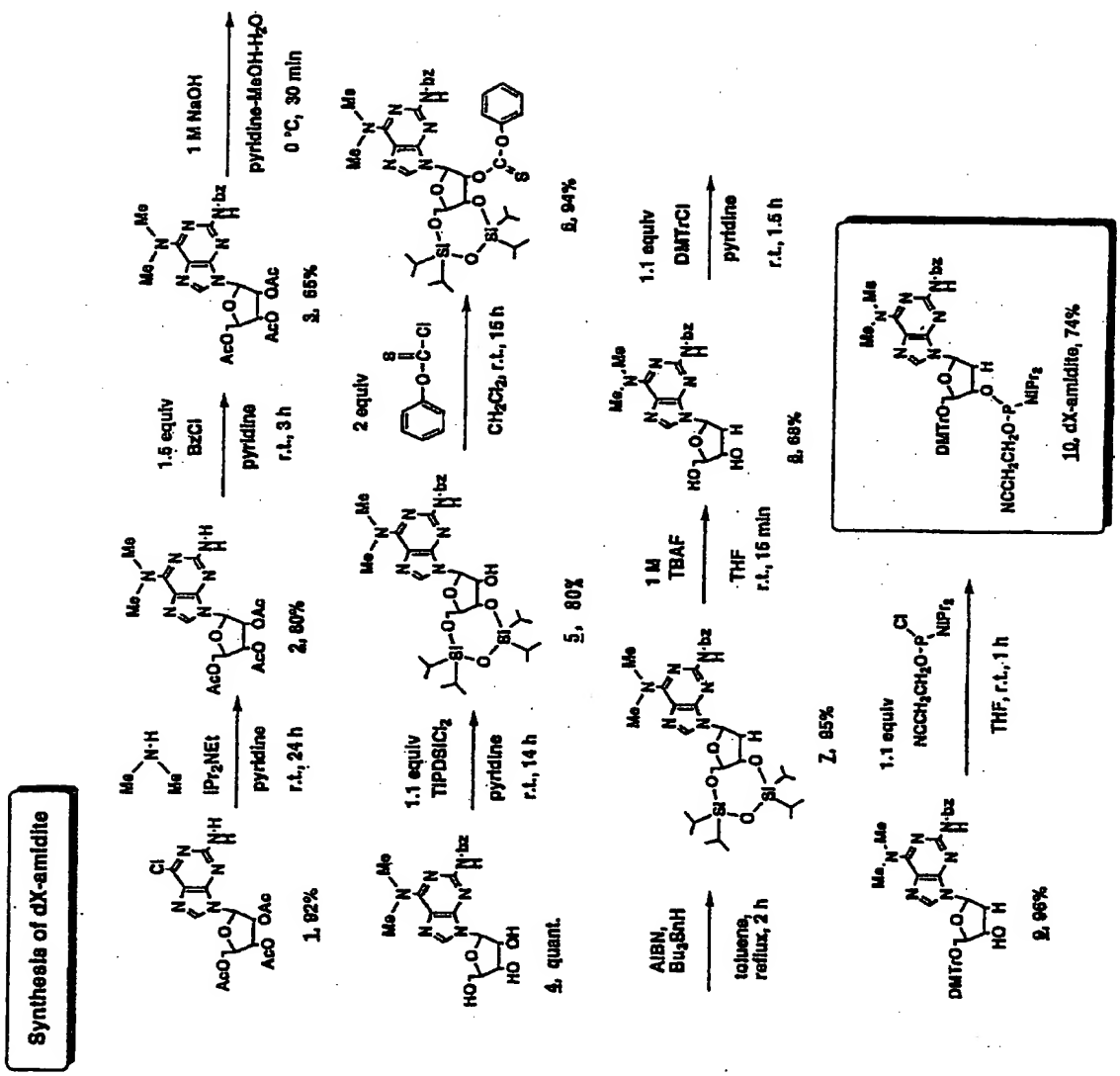




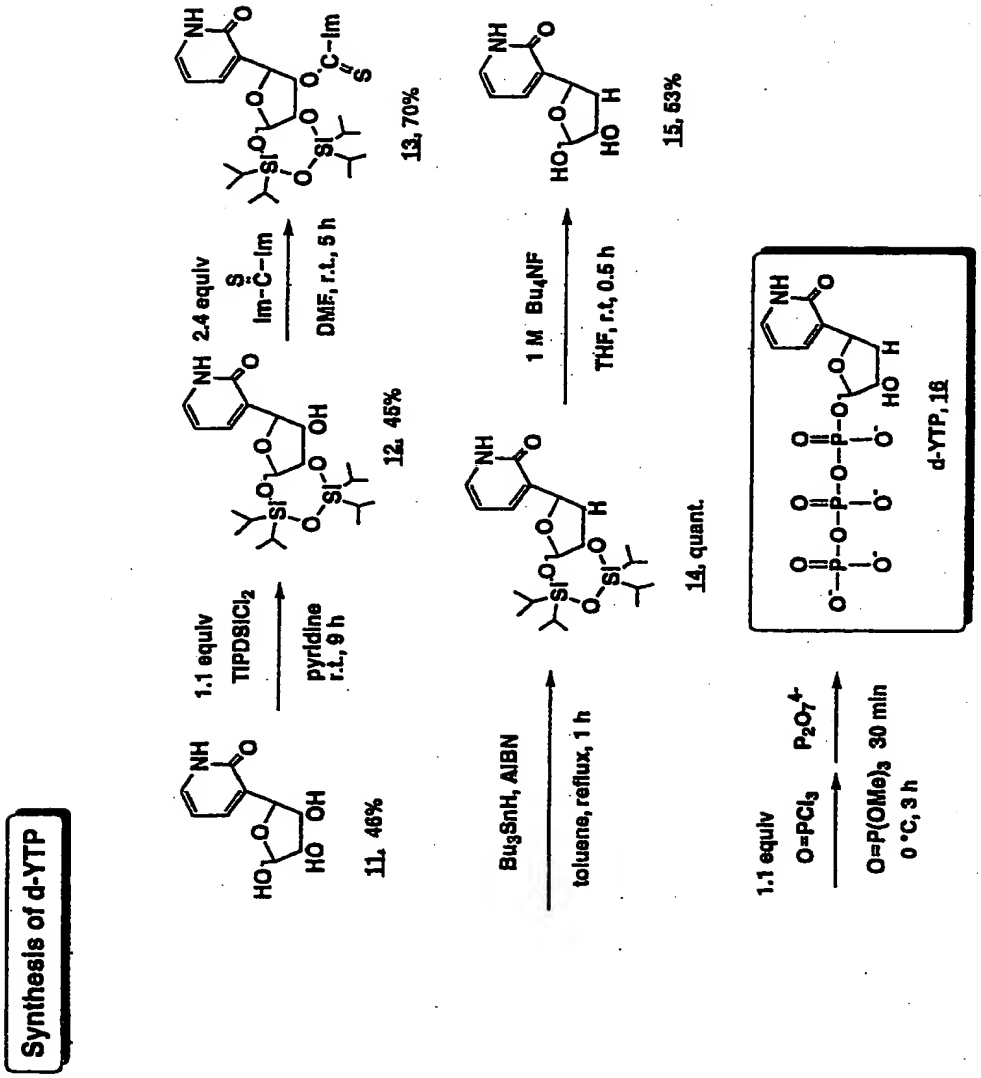


R=2-deoxy-β-D-ribpfuranosyl

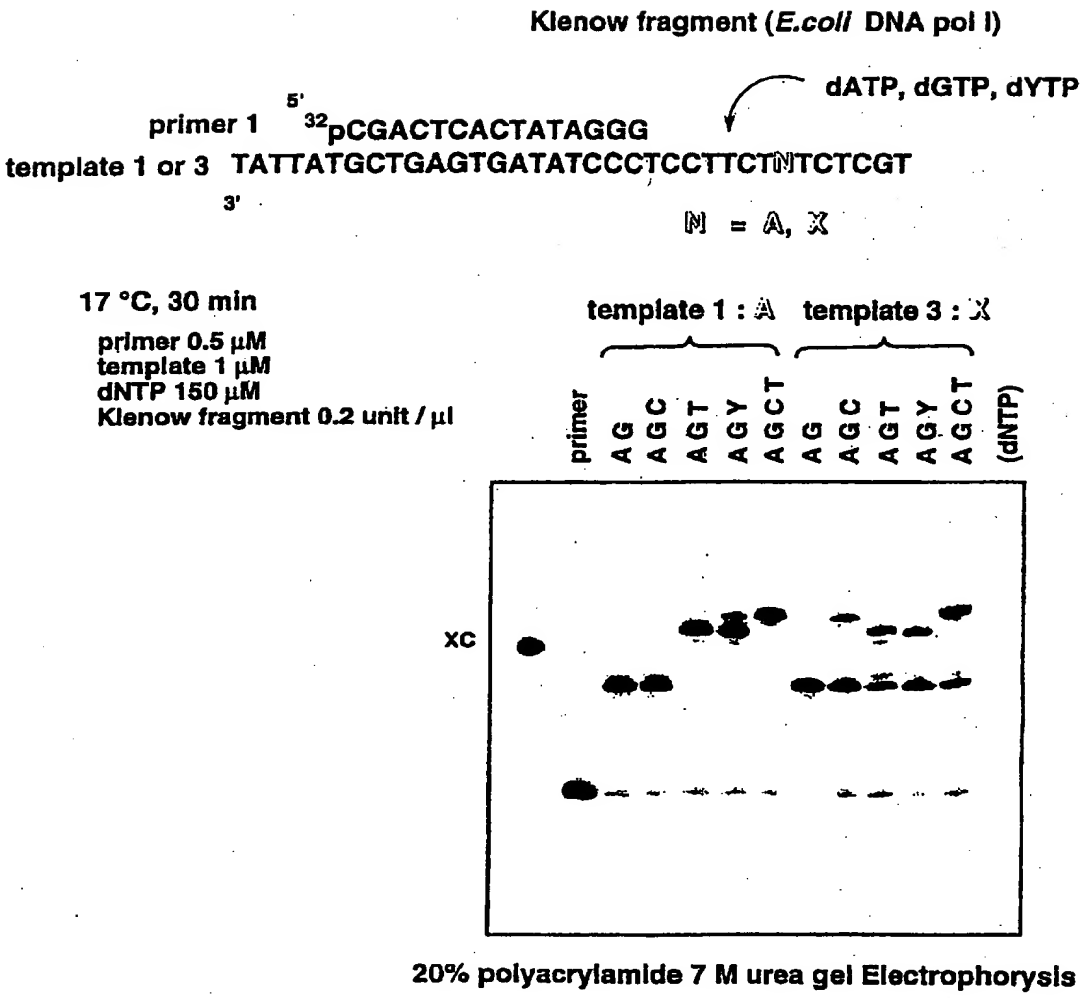
[Fig. 2]


Synthesis of dX-amidite
1, 92%
2, 80%
3, 65%
4, quant.
5, 80%
6, 94%
7, 85%
8, 68%
9, 96%
10, dX-amidite, 74%
iPr2NEt
pyridine
r.t., 24 h
1.5 equiv
BzCl
pyridine
r.t., 3 h
1 M NaOH
pyridine-MeOH-H2O
0 °C, 30 min
1.1 equiv
TIPDSiCl2
pyridine
r.t., 14 h
2 equiv
CH2Cl2, r.t., 15 h
1 M
TBAF
THF
r.t., 15 min
1.1 equiv
DMTrCl
pyridine
r.t., 1.5 h
AIBN,
Bu3SnH
toluene,
reflux, 2 h
1.1 equiv
NCCH2CH2O-P(Cl)NiPr2
THF, r.t., 1 h

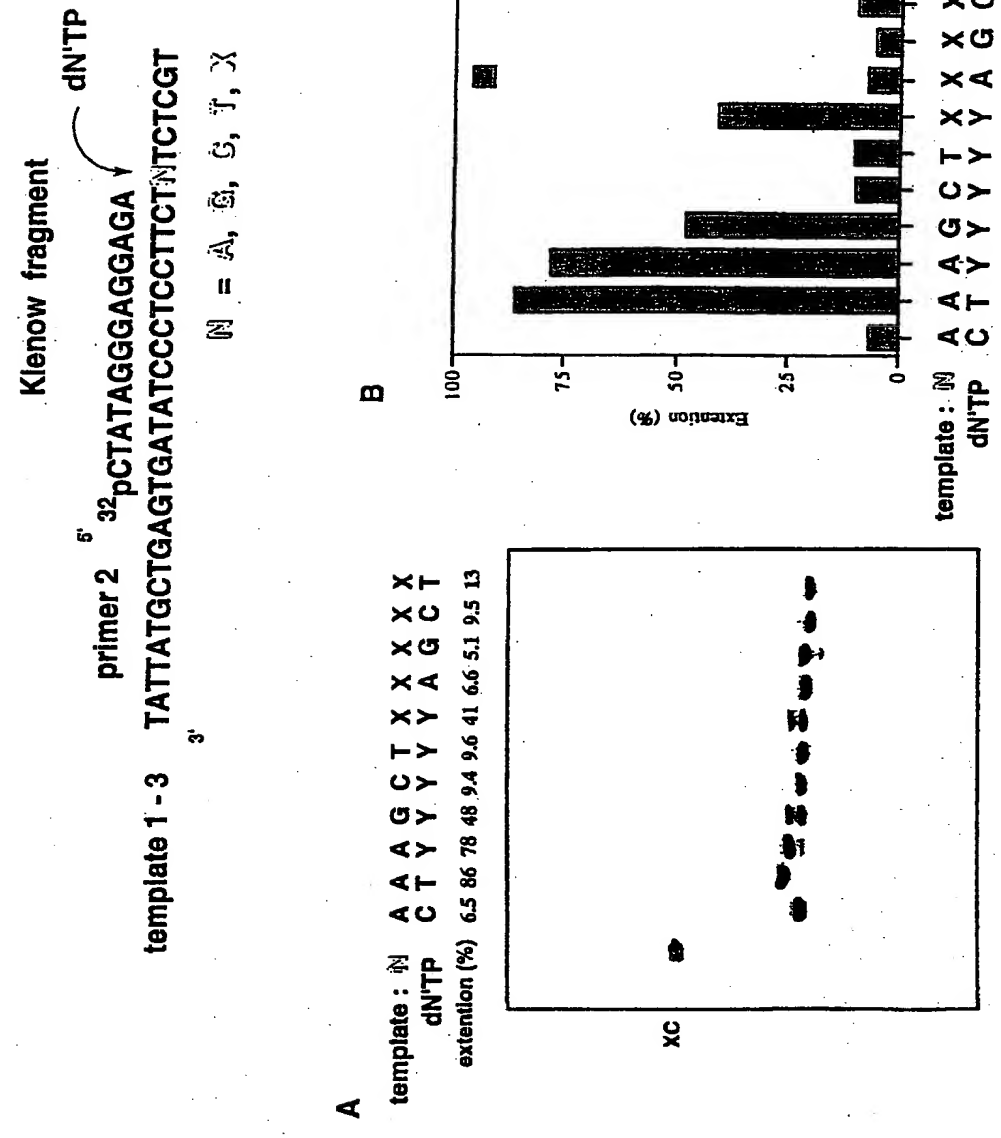
[Fig. 3]


Synthesis of d-YTP
1.1 equiv
TIPDSiCl2
pyridine
r.t., 9 h
11, 46%
12, 45%
2.4 equiv
Im-C(=S)-Im
DMF, r.t., 5 h
13, 70%
Bu3SnH, AIBN
toluene, reflux, 1 h
14, quant.
1 M Bu4NF
THF, r.t, 0.5 h
15, 53%
1.1 equiv
O=PCl3
P2O7 4-
O=P(OMe)3
0 °C, 3 h
30 min
d-YTP, 16

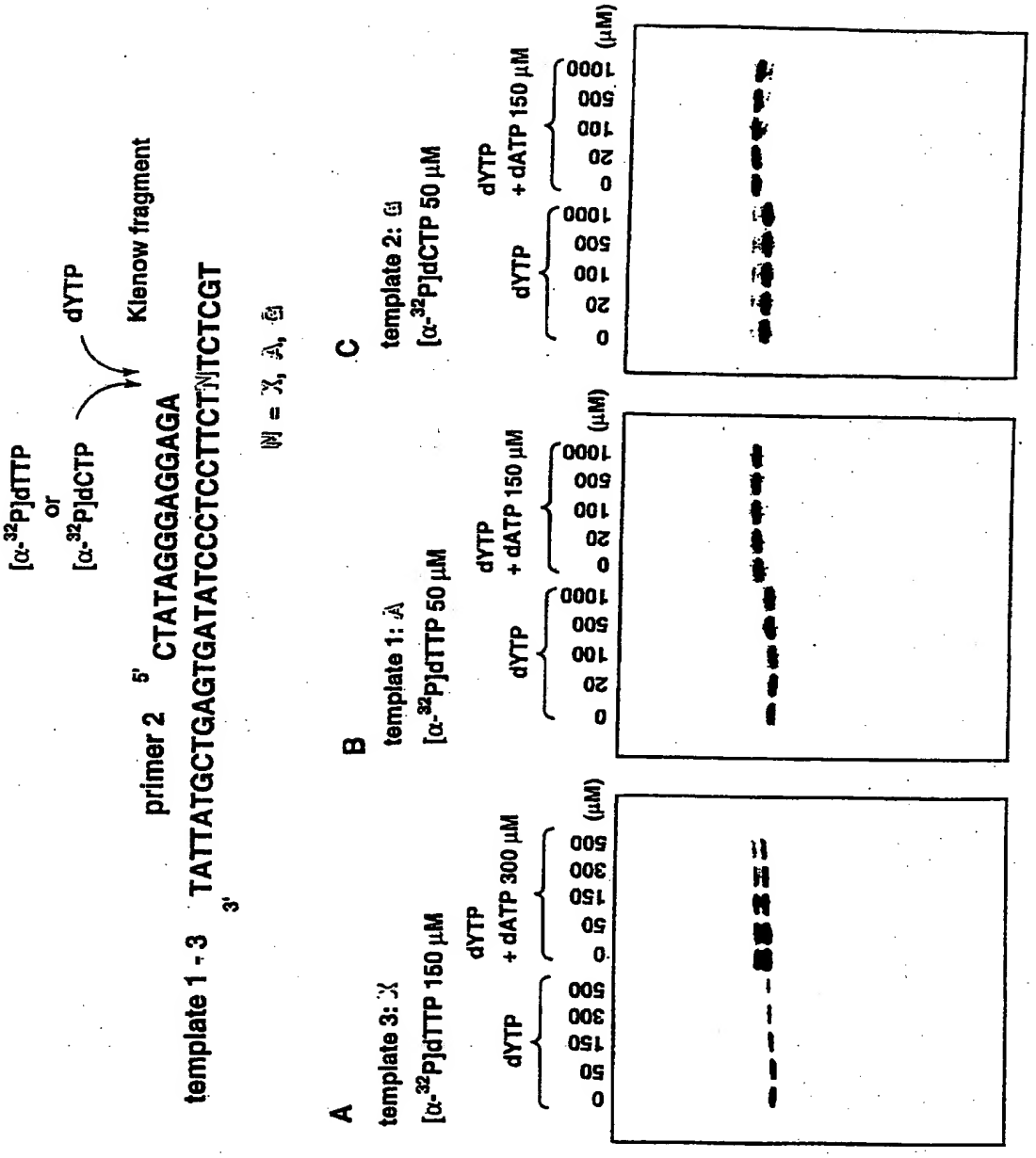
[Fig. 4]


Klenow fragment (E.coli DNA pol I)
dATP, dGTP, dYTP
5'
primer 1 32pCGACTCACTATAGGG
template 1 or 3 TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGT
3'
N = A, X
17 °C, 30 min
primer 0.5 μM
template 1 μM
dNTP 150 μM
Klenow fragment 0.2 unit / μl
template 1 : A
template 3 : X
primer
A
AG
AGC
AGT
AGY
AGCT
A
AG
AGC
AGT
AGY
AGCT
(dNTP)
XC
20% polyacrylamide 7 M urea gel Electrophorysis

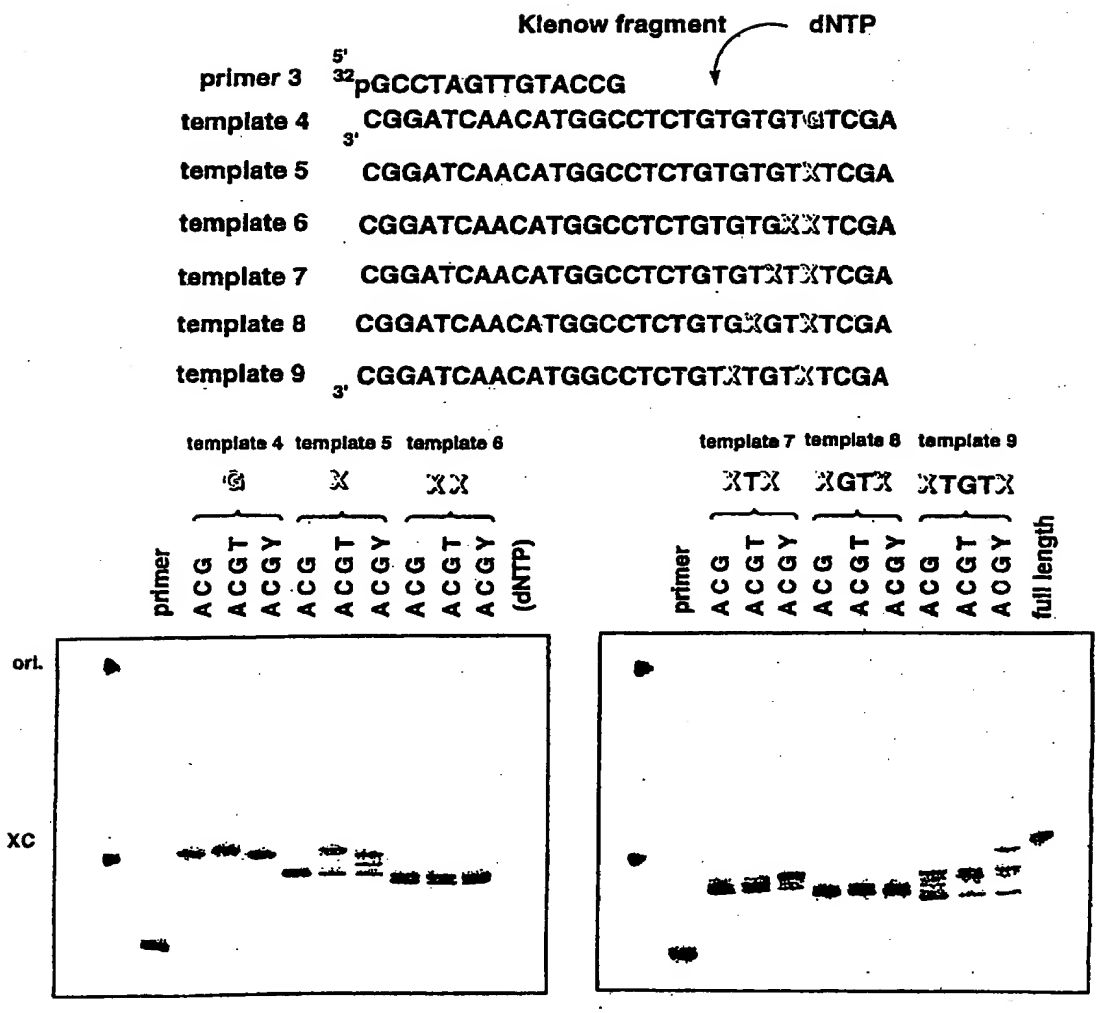
[Fig. 5]


Klenow fragment
dN'TP
primer 2 5' 32pCTATAGGGAGGAGA
template 1 - 3 TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGT 3'
N = A, G, T, X
A
template : N A A A G C T X X X X X
dN'TP C T Y Y Y Y Y A G C T
extention (%) 6.5 86 78 48 9.4 9.6 41 6.6 5.1 9.5 13
XC
B
Extention (%)
100
75
50
25
0
template : N A A A G C T X X X X X
dN'TP C T Y Y Y Y Y A G C T

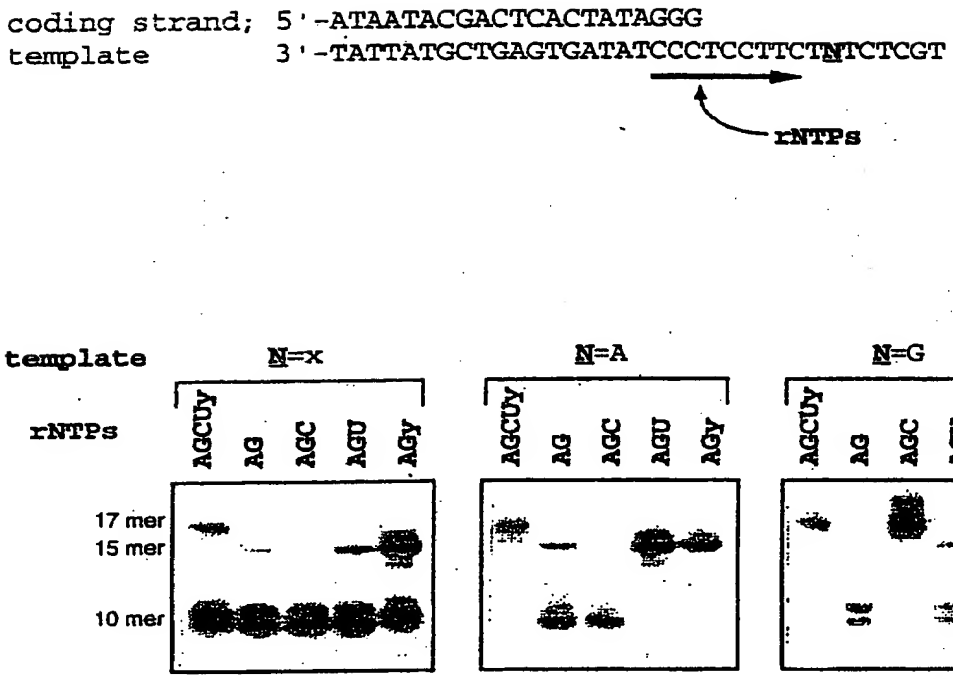
[Fig. 6]


[α-32P]dTTP
or
[α-32P]dCTP
dYTP
Klenow fragment
primer 2 5' CTATAGGGAGGAGA
template 1 - 3 3' TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGT
N = X, A, G
A
template 3: X
[α-32P]dTTP 150 µM
dYTP + dATP 300 µM
0 50 150 300 500 (µM)
B
template 1: A
[α-32P]dTTP 50 µM
dYTP + dATP 150 µM
0 20 100 500 1000 (µM)
C
template 2: G
[α-32P]dCTP 50 µM
dYTP + dATP 150 µM
0 20 100 500 1000 (µM)

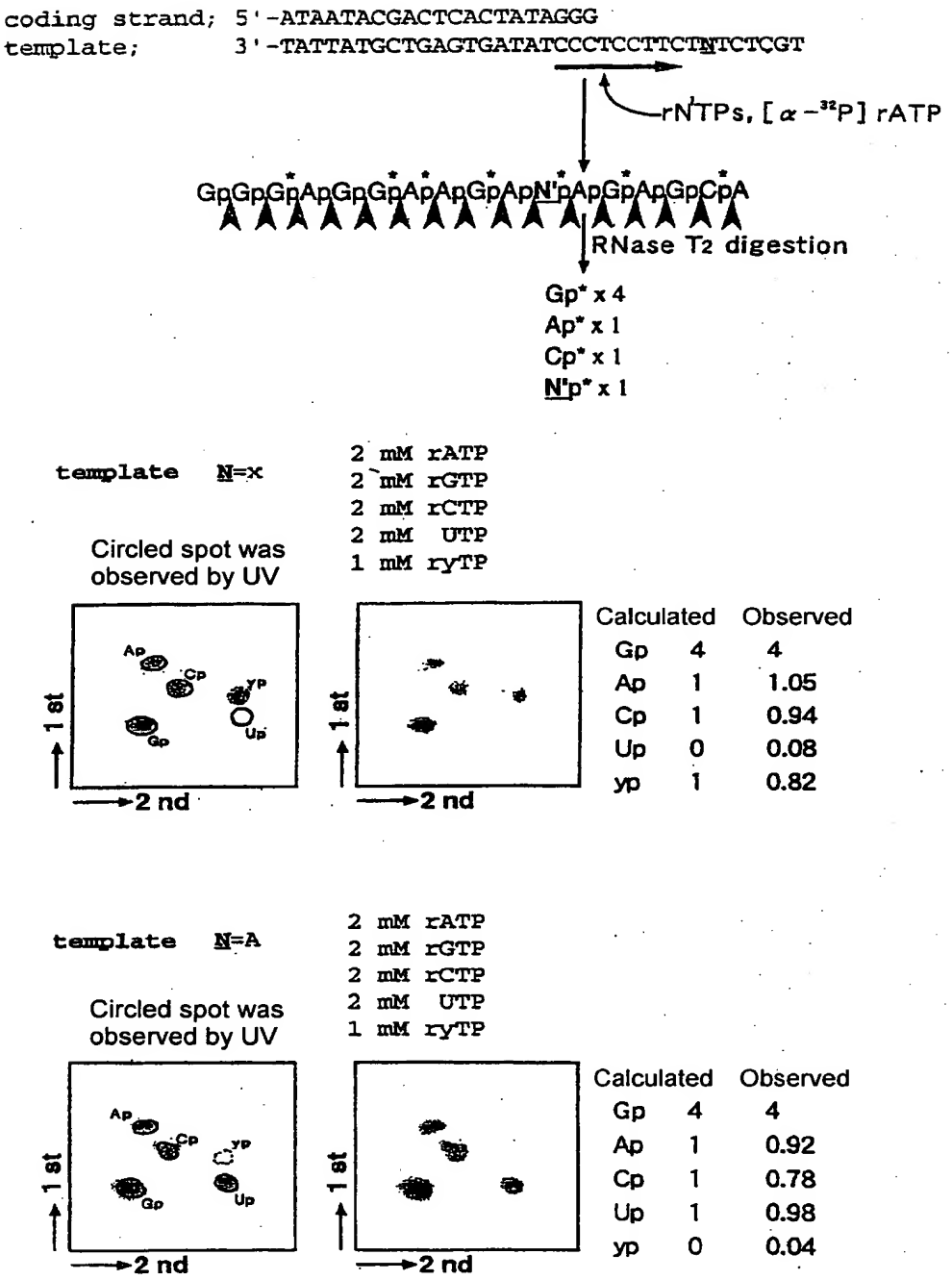
[Fig. 7]


Klenow fragment
dNTP
primer 3
5'
32pGCCTAGTTGTACCG
template 4
3'
CGGATCAACATGGCCTCTGTGTGTGTCGA
template 5
CGGATCAACATGGCCTCTGTGTGTXTCGA
template 6
CGGATCAACATGGCCTCTGTGTGXXTCGA
template 7
CGGATCAACATGGCCTCTGTGTXTXTCGA
template 8
CGGATCAACATGGCCTCTGTGXGTXTCGA
template 9
3'
CGGATCAACATGGCCTCTGTXTGTXTCGA
template 4
template 5
template 6
template 7
template 8
template 9
primer
A C G
A C G T
A C G Y
(dNTP)
full length
ori.
XC

[Fig. 8]


coding strand; 5'-ATAATACGACTCACTATAGGG
template 3'-TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGT
rNTPs
template
N=x
N=A
N=G
rNTPs
AGCUy
AG
AGC
AGU
AGy
17 mer
15 mer
10 mer

[Fig. 9]


coding strand; 5'-ATAATACGACTCACTATAGGG
template; 3'-TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGT
rNTPs, [α-32P] rATP
GpGpGpApGpGpApApGpApN'pApGpApGpCpA
RNase T2 digestion
Gp* x 4
Ap* x 1
Cp* x 1
N'p* x 1
template N=x
2 mM rATP
2 mM rGTP
2 mM rCTP
2 mM UTP
1 mM ryTP
Circled spot was observed by UV
1 st
2 nd
Calculated Observed
Gp 4 4
Ap 1 1.05
Cp 1 0.94
Up 0 0.08
yp 1 0.82
template N=A
2 mM rATP
2 mM rGTP
2 mM rCTP
2 mM UTP
1 mM ryTP
Circled spot was observed by UV
1 st
2 nd
Calculated Observed
Gp 4 4
Ap 1 0.92
Cp 1 0.78
Up 1 0.98
yp 0 0.04

[Document] Abstract

[Abstract]

[Problem]

The present invention provides novel artificial nucleic acid base pair which does not form base pair with natural nucleic acid and forms selective base pair in themselves as well as being recognized by various polymerases. Further aspect of the present invention is to provide artificial nucleic acid, codon containing the same, nucleic acid molecule, non-natural gene and application thereof.

[Means for Solving the Problems]

The present invention provides novel artificial nucleic acid base pair capable of forming selective base pair by utilizing steric hindrance which can be recognized by polymerases such as DNA polymerase, and novel artificial genes. Further, the present invention relates to a method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance in the nucleic acid base part. More particularly, the present invention relates to a method for designing nucleic acid to construct selective base pair by utilizing steric hindrance to hinder construction of base pair with natural nucleic acid base part, and the said base pair can be recognized by polymerase.

[Chosen Drawing] None